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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : A61K 39/395, C07K 16/34, C07H 21/04, C12N 15/13 — A1

(11) International Publication Number: WO 98/55146

(43) International Publication Date: 10 December 1998 (10.12.98)

(21) International Application Number: PCT/US98/11328

(22) International Filing Date: 5 June 1998 (05.06.98)

(30) Priority Data:
60/048,645 5 June 1997 (05.06.97) US

(63) Related by Continuation (CON) or Continuation-in-Part (CIP) to Earlier Application
US 60/048,645 (CIP)
Filed on 5 June 1997 (05.06.97)

(71) Applicant *(for all designated States except US)*: JOHNS HOPKINS UNIVERSITY [US/US]; 720 Rutland Avenue, Baltimore, MD 21205 (US).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: KICKLER, Thomas, S. [US/US]; 4 Palmer Green, Baltimore, MD 21210 (US). KENNEDY, Sean, D. [US/US]; 3607 Keswick Road, Baltimore, MD 21211 (US). OKAMOTO, Naoaki [JP/JP]; Tokai University of Medicine, Division of Molecular Life Science, Dept. of Genetic Information, Bohseidai, Isehara, Kanagawa 259–11 (JP).

(74) Agents: CORLESS, Peter, F. et al.; Dike, Bronstein, Roberts & Cushman, 130 Water Street, Boston, MA 02109 (US).

(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, GW, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: IDENTIFICATION OF HUMAN HEAVY CHAIN ANTIBODY FRAGMENT DIRECTED AGAINST HUMAN PLATELET ALLOANTIGEN 1a(HPA–1a)

(57) Abstract

A human immunoglobulin heavy chain sequence capable of differentiating between the HPA–1a protein and the HPA–1b protein was isolated from a repertoire of heavy chain variable region genes from an alloimmunized individual. Additional isolation, analysis, and modification of this antibody fragment and similar anti–HPA–1 antibodies holds potential for understanding key aspects of the HPA–1 antigen–antibody interaction and for developing therapeutic applications.

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IDENTIFICATION OF HUMAN HEAVY CHAIN ANTIBODY FRAGMENT DIRECTED AGAINST HUMAN PLATELET ALLOANTIGEN 1a (HPA-1a)

CROSS-REFERENCE TO RELATED APPLICATION

This application is a continuation-in-part of U.S. provisional application number 60/048,645, filed June 5, 1997, which is incorporated herein by reference.

TECHNICAL FIELD OF THE INVENTION

The present invention relates to the identification of a human heavy chain antibody fragment to human platelet alloantigen-1a (HPA-1a), and more particularly to nucleotide and amino acid sequences identified herein as having the ability to inhibit platelet aggregation, and to the diagnostic and therapeutic uses to which such sequences may be placed.

BACKGROUND OF THE INVENTION

The human platelet antigens, HPA-1a (PI^{A1}) and HPA-1b (PI^{A2}), are determined by two allelic forms of the human platelet membrane glycoprotein (GP) IIIa gene. A polymorphism at nucleotide 196 results in a leucine (HPA-1a) or proline (HPA-1b) substitution at amino acid position 33 (Newman et al. (1989) *J. Clin. Invest.* 83:1778-81). It is not yet known if this substitution changes the function of the platelet membrane protein, this polymorphism can trigger neonatal alloimmmune thrombocytopenia (NAIT), post transfusion purpura (PTP) and contribute to platelet transfusion refractoriness in a subset of multiply transfused patients (Goldman et al. (1994) *Transfus. Med. Rev.* 13:123-31, Waotier et al. (1993) *Nouv. Rev. Fr. Hematol.* 35:171-178). In a recent study of NAIT in a Caucasian population, 78 percent of cases were caused by anti-HPA-1a alloantibody and 19 percent were caused by anti-HPA-5b (Br^a) (Mueller-Eckhardt et al. (1989) *Lancet* 1:363-6). Post transfusion purpura follows 7 to 10 days after blood or platelet transfusion and most often affects previously untransfused HPA-1a-negative (HPA-1b homozygous) multiparous women. It was recently suggested that all pregnant women should be tested for their HPA-1 system phenotype because HPA-1b homozygous have significantly higher risk for NAIT and purpura (Flug et al. (1994) *Br J. Haematol.* 86:1-5). However, not all HPA-1a

homozygous women make antibodies, thus DNA screening is not necessarily helpful, instead a screening test for antibody to HPA-1a may be a better test.

Phenotyping of HPA-1 system depends on human antisera containing alloantibody to HPA-1a. Unfortunately, these antisera are rare and typing is confused by the presence of antibodies against human leukocyte antibodies (HLA) and blood groups. An alternative approach, production of monoclonal antibodies through murine hybridoma techniques, has not been entirely satisfactory. Although one monoclonal antibody, SZ21, is known to recognize the HPA-1a form, it is expensive to purchase and is not able to be easily produced locally. In addition to the difficulty in differentiating the single amino acid difference in platelet membrane GP IIIa there is limited murine immune response to the human alloantigen system (Liu et al. (1992) *Br J Haematol* 81:113-7, Ryckewaert et al. (1992) *J. Lab. Clin. Med.* 119:52-6, Weiss et al. (1995) *Tissue Antigens* 46:374-81).

It has recently become possible to clone and express certain human antibody fragments using phage display technology (Orlandi et al. (1989) *Proc. Natl. Acad. Sci. USA* 86:3833-37, McCafferty (1990) *Nature* 348:552-4, Marks et al. (1991) *J. Mol. Biol.* 222:581-97, Clackson et al. (1991) *Nature* 352:624-8). Repertoire cloning of antibody fragments has been used to isolate certain human antibodies directed at a number of soluble, purified antigens, including tetanus toxoid (Mullinax et al. (1990) *Proc. Natl. Acad. Sci USA* 87:8095-99), human immunodeficiency virus surface antigen (Burton et al. (1991) *Proc. Natl. Acad. Sci USA* 88:10134-7), hepatitis B virus surface antigen (Zebedee et al. (1992) *Proc. Natl. Acad. Sci. USA* 89:3175-9) and human blood group antigens (Marks et al. (1993) *Bio/Technology* 11:1145-9, Siegel et al. (1994) *Blood* 83:2334-44, Hughes-Jones et al. (1994) *Br J Haematol* 88:180-6.

SUMMARY OF THE INVENTION

The present invention relates to the identification and elucidation of a recombinant heavy chain antibody fragment specific for HPA-1a, and more particularly to nucleotide and amino acid sequences identified herein as having the ability to inhibit platelet aggregation, and to the diagnostic and therapeutic uses to which such sequences may be placed. Specifically, the present invention provides isolated polypeptides and nucleic acid sequences encoding such polypeptides useful for development of therapeutic agents for treating or preventing platelet aggregation. In

addition, such polypeptides and DNA sequences are useful for phenotyping of HPA-1 system to identify HPA-1b homozygote. These polypeptides and sequences are useful for typing large numbers of platelet samples from pregnant women to reduce NAIT and to determine risk associated with myocardial infarction. Additional isolation, analysis, and modification of such antibody fragments and similar anti-HPA-1 antibodies holds potential for understanding key aspects of the HPA-1 antigen-antibody interactions.

Preferred polypeptides of the invention bind with high specificity to HPA-1a. More specifically, preferred polypeptides of the invention will bind HPA-1a at least about two-fold greater than binding of HPA-1b, more preferably will bind HPA-1a at least about three-fold greater than binding of HPA-1b, and still more preferably will bind HPA-1a at least about 3.5- or four-fold greater than binding of HPA-1b. See, for instance, the results of Example 3 which follows.

Preferred polypeptides of the invention are of human origin, preferably completely of human origin (distinguished from chimerics), and thus can be used in therapeutic applications with a lower potential for antigenic reactivity in humans.

Preferred polypeptides have high binding affinity for HPA-1a, preferably about the same or more than ML1, as measured in a standard *in vitro* binding assay. For example, antibody association (Ka) and dissociation (Kd) values for a polypeptide of the invention can be determined by ELISA or surface plasman resonance (i.e., BIACore assays (See e.g., the assays disclosed in Harlow, *Antibodies: A Laboratory Manual*, CSH Publications, NY (1988); Asubil et al., *Current Protocols in Molecular Biology*, John Wiley & Sons, NY (1989); Altcuhuh et al., *Biochem.*, 31:6298 (1992); and the BIACore method disclosed by Pharmacia Biosensor).

Polypeptides and nucleic acids of the invention are particularly useful in inhibiting the binding of fibrinogen to blood platelets, inhibiting the aggregation of blood platelets and treatment and prevention of thrombus formation or embolus formation, especially by inhibiting platelet activation by thrombin. The invention thus provides methods for treatment and prevention of conditions involving platelet activation and aggregation, such as arterial or venous cardiovascular disorders, cerbrovascular thromboembolic disorders such as myocardial infarction, stroke, unstable angina, ischemic sudden death, transient ischemic attack, atherosclerosis, or

undesired reocculsion and restonosis after cardiac angioplasty or other cardiac surgery, or other surgical procedures.

Polypeptides of the invention also used for identification and diagnosis of subjects suffering from or susceptible to thrombotic disease. In particular, preferred polypeptides of the invention can be employed to identify (e.g. by flow cytometry or other means) the coronary thrombosis risk factor GPIIIa leu33. Hence, large groups can be screened for the risk factor to identify subjects at increased risk for thrombosis and related disorders.

As discussed above, preferred polypeptides of the invention bind to the N-terminus of GPIIIA, where it has been found that autoantigens involved in HIV-induced immune thrombocytopenia are present. Preferred polypeptides of the invention thus can be used to identity and isolate substantially pure autoantigens that are involved in HIV-induced immune thrombocytopenia. For example, polypeptides of the invention can be used in a column format to selectively elute the autoantigens from a biological sample.

Such isolated autoantigens or polypeptides of the invention may be used e.g. for diagnostic testing of a body fluid (e.g., plasma or serum) or tissue (e.g., a biopsy sample). For example, the autoantigens can be used in a suitable immunological format such as an ELISA format to detect or diagnose immune thrombocytopenia.

Polypeptides of the invention also may be employed to generate anti-idiotypic antibodies for use in various therapeutic methods. For example, such anti-idiotypic antibodies can be administered to subjects suffering from HIV-induced immune thrombocytopenia to provide a substantially more specific treatment than current human immunoglobulin therapies.

In the therapeutic methods of the invention, one or more polypeptides or nucleic acids of the invention may be administered as the sole therapeutic agent(s), or the one or more polypeptides or nucleic acids can be administered in combination with other therapeutics, e.g. either co-administered in a cocktail format with other pharmaceutical agents or as a directly (covalently) linked carrier of other pharmaceuticals, particularly anti-coagulant agents such as heparin or warfarin; antiplatelet agents such as ticlopidine, piroxicam or aspirin; or a thrombin inhibitor

such as a hirudin, a boropeptide, or argatroban; or a thromboyltic agent such strepokinase, tissue plasminogen activator (t-PA) or urokinase.

Other aspects of the invention are discussed infra.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGURE 1 depicts the binding of the ML1 clone as analyzed by flow cytometry. Expression phage ML1 clone was added to platelet from a HPA-1a and HPA-1b homozygous donor. Biotinylated anti-M13 was added and then a streptavidin FITC conjugate. Samples were gated with a phycoerythrin labeled antibody to platelet protein CD41. Negative control sample was an expression phage that does not bind to human platelets. The histogram B demonstrates binding of the ML1 clone to HPA-11 platelets while the histogram A demonstrates no binding of the ML1 clone.

FIGURE 2 depicts the HPA-1a binding of a human heavy chain antibody fragment. The nucleic acid sequence (SEQ. ID NO: 1) is shown above the corresponding amino acid sequence (SEQ. ID NO:2). The region designation is shown by arrow as follows: FR=Framework and CDR=Complementary determining region.

FIGURE 3 depicts the ability of clone ML1 to inhibit platelet aggregation.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to the identification and elucidation of a recombinant heavy chain antibody fragment specific for HPA-1a, and more particularly to nucleotide and amino acid sequences identified herein as having the ability to inhibit platelet aggregation, and to the diagnostic and therapeutic uses to which such sequences may be placed. Such sequences bind to HPA-1a, the GST N-terminal region of GP111a (glycoprotein 111a), and not HPA1b platelets and inhibit a normal aggregation response.

Recent advances in molecular biology have facilitated the isolation and in vitro production of antibodies by creating libraries of recombinant immunoglobulin Fab fragments on the surface of bacteriophage particles. These approaches have been applied to produce the recombinant antibodies that can bind to small haptens as well as complex protein molecules. These methods are proving especially valuable for the study

and isolation of antibodies from humans for whom lymphocyte immortilization techniques are still limited.

The present invention describes the development of a heavy chain variable region antibody fragment from the blood of an alloimmunized individual. A mix of heavy chain variable region genes were amplified from cDNA prepared from mRNA derived from the lymphocytes of an anti-HPA-1a positive individual. Amplification was accomplished through degenerate PCR primers. These primers were designed to cover most of the repertoire of heavy chain immunoglobulin subclass sequence (Marks et al. (1991 *Eur. J. Immunol.* 21:985-91) and appeared to amplify target immunoglobulin heavy chain cDNA with high specificity. In these experiments, only heavy chain genes were expressed on the surface of phage.

Most of recombinant phage antibodies studies have utilized either double domain antibodies (Fab fragment), or single chain Fv constructs with heavy and light chain variable regions linked by linker sequence. However, several recent studies have suggested the ability of single domain antibodies to bind with reasonable affinity to whole protein molecules (Ward et al. (1989) *Nature* 341:544-6, (Williams et al. (1993 *Transgene* 1:113-23). The recombinant antibody fragment described here also appears to have acceptable affinity and specificity to HPA-1a protein despite the potential limitations due to the presence of only heavy chain sequence. We tried to produce ScFv antibodies by direct PCR linkage of heavy and light chain using cohesive linker sequence, but only ScFv antibodies with low specificity to HPA-1a protein were selected from the library (data not shown). This low specificity may have been due to a poor repertoire of linked heavy chain and light chain from the direct PCR link reactions. To select the ScFv antibody with high specificity for HPA-1a protein, chain shuffling (Marks et al. (1992) *Bio/Technology* 10:779-83) of this heavy chain gene using light chain library and pre-absorption panning method using HPA-1b protein might be more effective.

HPA-1a and HPA-1b are two allelic forms of the platelet membrane glycoprotein (GP) IIIa gene which differ by a single amino acid. The present invention provides *inter alia* a recombinant heavy chain antibody fragment capable of distinguishing between HPA-1a and HPA-1b. For example, the human platelet alloantigens, HPA-1a (PI^{A1}) and HPA-1b (PI^{A2}), are responsible for most cases of post transfusion purpura (PTP) and

neonatal alloimmune thrombocytopenia (NAIT) in the Caucasian population. A preferred antibody fragment was isolated from the lymphocytes of an immunized individual through the use of a phage display library system. The recombinant antibody fragment reacted with human platelet lysates from HPA-1a homozygous donor, HPA-1a form of recombinant N-terminal GP IIIa and intact HPA-1a platelets, but did not react with platelet lysate from HPA-1b homozygous donor, reduced HPA-1a form of platelet GP IIIa or other platelet glycoproteins. This HPA-1a specific human antibody fragment works in ELISA and flow cytometry, common assays that can identify HPA-1b homozygous individuals and have, therefore, a higher risk for developing NAIT and PTP. This demonstrates that selection of recombinant antibody using phage display library of human antibody fragment offers a promising alternative to hybridoma technology for the production of human antibodies against human alloantigens and holds potential as a technique for developing a human antibody useful in therapeutic applications.

The recombinant heavy chain antibody fragment did not react with reduced platelet GP IIIa, HPA-1a form, suggesting that the antibody fragment recognized a HPA-1a containing conformation determined by disulfide bonds. This heavy chain antibody fragment was also effective and specific in flow cytometric immunofluorescence assays using intact platelets. These results indicate that this heavy chain antibody fragment can substitute in many widely used typing techniques, such as ELISA, flow cytometry and MAIPA (Kiefel et al. (1987) *Blood* 70:1722-6). This recombinant heavy chain antibody fragment and other polypeptides of the invention should have several significant advantages for these assays. The phage antibody is easily produced by bacterial culture, which is less expensive and technically easier than eukaryotic cell culture. Furthermore, significant quantities can be produced rapidly during overnight culture as opposed to the many days usually required for eukaryotic cell culture. Most significantly, phage antibody technology also allows the use of molecular techniques to modify the DNA sequence of the antibody fragment gene. Thus, it is possible to continue to improve affinity and specificity, to add the Fc sequence for MPHA assay (Shibata et al. (1981) *Vox Sang* 41:25-31) or for second signal antibody binding, or conjugate marker molecule. These approaches could produce a stable and plentiful source of specific HPA-1a and

HPA-1b antibody fragments for typing large numbers of platelet samples in screening assays.

The heavy chain antibody fragment described here is separable from the host phage particle. Since the heavy chain antibody fragment sequences produced by this method are of human origin only, there should be a lower potential for antigenic reactivity in humans. As a consequence, this approach may be useful for the construction of therapeutic antibodies that can block platelet function (Van Leeuven et al. (1984) *Vox Sang* 47:280-6). Since the amino acids of the antibody fragments are human, making them less immunogenic than antibodies that are human-murine chimeras, human antibody fragments that bind to important epitopes carried on platelet glycoprotein IIIa are an improvement over those humanized murine monoclonals that are currently being used to inhibit platelet function (Marks (1996) *N. Engl. J. of Med.* 335:730-3). Comparison of the DNA sequence of the anti-HPA-1a heavy chain gene reported here with a previously described antibody gene has shown multiple differences at the nucleotide and amino acid level though both recognize the same antigen. Analysis of these sequences could lead to a better molecular understanding of antigen-antibody interactions and could contribute to the development of the molecular design of higher affinity antibodies

Preferably, the compounds of the present invention are polypeptides, such as SEQ. ID NO: 2, QVQLQESGPGLVKPSETLSLTCNVSGGSISSYYWSWIRQPPGKG LEWLGYLYNSGSTIYSPALESRATISVDTSKNQFSLKLSSVTAADTAVYYCARP EPYSSGWYRGIFDAFDIWGQGTKVTVLG and its derivatives. It is well known in the art that conservative substitutions may be made in the amino acid sequence without losing functionality. For example, substitutions are well known and are based upon the charge and structural properties of each amino acid. Such functionality equivalent peptides are also encompassed in the present invention.

Thus, preferred polypeptides of the invention include those that have substantial sequence identity (homology) to SEQ ID NO:2 (shown in Fig. 2). More particularly, preferred polypeptides include those that have at least about 70 percent homology (sequence identity) to SEQ ID NO:2, preferably at least about 80 percent homology (sequence identity) to SEQ ID NO:2, still more preferably at least about 85, 90, 95, 97 or 98 percent homology to SEQ ID NO:2. Sequence identity or homology

with respect to SEQ ID NO:2 refers to herein as the percentage of amino acids of a polypeptide is identical with SEQ ID NO:2, after introducing any gaps necessary to achieve the maximum percent homology. A polypeptide of the invention typically will comprise at least about 20 amino acids, more typically at least about 30, 40, 50 or 60 amino acids.

Preferably, the isolated nucleic acid molecule provided by the present invention, such as SEQ. ID NO.1 atggcccaggtgcagctgcaggagtcgggcccaggactggtgaagcc ttcggagaccctgtccctcacctgcaatgtctctggtggctccatcagtagttactactggagttggatccggcagcccccag gaagggactggagtggcttgggtatttgtataacagtggcagcaccatctacagccccgccctcgagagtcgagccacca tatccgtagacacgtccaagaaccagttctcccctgaagctgagctctgtgaccgccgcagacacagctgtgtattactgtg cgaggcccgaaccttatagcagtggctggtaccggggcatttttgatgcttttgatatctggggccaagggaccaaggtcac cgtcctaggt, encodes polypeptides, allelic variants, or analogs, including fragments of the invention. Specifically provided are DNA molecules for use in securing expression of a polypeptide have the biological activity of altering platelet function.

Nucleic acids of the invention preferably are of a length sufficient (preferably at least about 20, 30, 40, 50, 60, 70, 80, 90, 100, 120 or 150 base pairs) to bind to the sequence of SEQ ID NO:1 (shown in Fig. 2) under the following moderately stringent conditions (referred to herein as "normal stringency" conditions): use of a hybridization buffer comprising 20% formamide in 0.8M saline/0.08M sodium citrate (SSC) buffer at a temperature of 37°C and remaining bound when subject to washing once with that SSC buffer at 37°C.

More preferably, nucleic acids of the invention (preferably at least about 20, 30, 40, 50, 60, 70, 80, 90, 100, 120 or 150 base pairs) will bind to the sequence of SEQ ID NO:1 under the following highly stringent conditions (referred to herein as "high stringency" conditions): use of a hybridization buffer comprising 30% formamide in 0.9M saline/0.09M sodium citrate (SSC) buffer at a temperature of 45°C and remaining bound when subject to washing twice with that SSC buffer at 45°C.

Preferred nucleic acids of the invention also will have substantial sequence identity (homology) to SEQ. ID NO:1. More particularly, preferred nucleic acids will comprise a sequence that has at least about 70 percent homology (sequence identity) to SEQ ID NO. 1, more preferably about 80 percent or more homology to SEQ ID

NO. 1, still more preferably about 85, 90, 95, 97 or 98 percent or more homology to SEQ ID NO. 1. Sequence identity or homology with respect to SEQ ID NO:1 refers to herein as the percentage of base sequences of a nucleic acid that is identical with SEQ ID NO:1, after introducing any gaps necessary to achieve the maximum percent homology.

Nucleic acids of the invention are isolated, which means that a given nucleic acid usually constitutes at least about 0.5%, preferably at least about 2%, and more preferably at least about 5% by weight of total nucleic acid present in a given fraction. A partially pure nucleic acid constitutes at least about 10%, preferably at least about 30%, and more preferably at least about 60% by weight of total nucleic acid present in a given fraction. A pure nucleic acid constitutes at least about 80%, preferably at least about 90%, and more preferably at least about 95% by weight of total nucleic acid present in a given fraction.

"Thrombus" shall mean an aggregation of blood factors, primarily platelets and fibrin with entrapment of cellular elements, frequently causing vascular obstruction at the point of its formation. Thrombus is meant to encompass clot formation other than when normal physiological coagulation occurs and the inhibition of platelet aggregation would be harmful to the patient. The compositions of the present invention may be useful for treating or preventing thrombus and/or platelet-related problems. Thrombus and platelet-related syndromes and diseases, include, but are not limited to, acute myocardial infarction, acute stroke, postsurgical cardiac surgery, post vascular graft surgery, thrombocytosis, anti-platelet-related autoimmunity, anti-thrombus formation, post transfusion purpura, and neonatal alloimmune thrombocytopenia.

Also, as used herein, the term "antibody" refers to immunologically active antibody fragments, such as the preferred antibody ML1, as well as whole immunoglobulins that can preferably selectively bind HP-1a.

Post transfusion purpura (PTP) and neonatal alloimmune thrombocytopenia (NAIT) are the primary disorders in which platelet-specific alloimmunization is involved. A major source for these two disorders is due to the HPA-1 platelet alloantigen system. Approximately 70-80% of the cases of neonatal alloimmune thrombocytopenia(NAIT) in the Caucasian population are linked to the HPA-1

polymorphism. Like Rh hemolytic disease of the newborn, NAIT is due to alloimmunization of the mother (in most case, a mother with HPA-1b homozygous type of platelets) with an incompatible alloantigen (HPA-1a form) on the fetus's platelets. However, unlike Rh disease of the newborn, approximately 50% of babies with NAIT are first births and 97% of subsequent pregnancies are affected. At present, the diagnosis of HPA-1-induced NAIT is made only after a thrombocytopenic infant is born.

To reduce this NAIT, it would be advantageous to screen the pregnant women to identify the subset which are homozygous for HPA-1b. Currently, determination of the HPA-1 status of patients is contingent upon the availability of human anti-HPA-1a antisera. Anti-HPA-1a antisera, however, are rare and not routinely available in hospitals. In addition, their use is confounded by the presence of anti-HLA or anti-ABO alloantibodies which can interfere with the assay by giving false positives. In order to improve specificity, mouse monoclonal antibodies against HPA-1a protein have been produced using conventional hybridoma technology. Unfortunately, except for SZ21, these antibodies do not differentiate the allotype when used with intact platelets (Liu et al. (1992) *Br J Haematol* 81:113-7, (Ryckewaert et al. (1992) *J. Lab. Clin. Med.* 119:52-6, Weiss et al. (1995) *Tissue Antigens* 46:374-81). For this reason, no clinical studies have been reported using such monoclonal antibodies. An alternative approach, using DNA techniques to look at the genes for the typing of platelet antigens has been reported by several groups (Liu et al. (1992) *Br J Haematol* 81:113-7, Ryckewaert et al. (1992) *J. Lab. Clin. Med.* 119:52-6, Weiss et al. (1995) *Tissue Antigens* 46:374-81). Platelet typing using PCR technology has demonstrated impressive accuracy, but the cost for this method is high and the method is technically demanding since strict guidelines must be observed to prevent sample cross contamination. In reality, it may be difficult to justify DNA typing of large numbers of platelet samples to screen pregnant women given the incidence of NAIT. These limitations have led to the desire to use well established immunoassays in combination with improved methods for producing specific antibodies.

Sequences of the present invention useful for phenotyping individuals susceptible to developing myocardial infarction can be suitably obtained as follows.

Amplification of immunoglobulin heavy chain variable regions

Peripheral blood mononuclear cells (PBMC) were isolated from a patient producing anti-HPA-1a alloantibody using Histopaque-1077 (Sigma Diagnostic, St.

Louis, MO). mRNA was prepared using oligo-dT affinity spin column (FastTrack mRNA Isolation Kit, Invitrogen, San Diego, CA) from 1 x 10^8 PBMC. First-strand cDNA was synthesized from mRNA with reverse transcriptase using an oligo-dT primer (cDNA Cycle Kit, Invitrogen, San Diego, CA).

For polymerase chain reaction (PCR) amplification of the immunoglobulin heavy chain gene repertoire, the oligonucleotide primers listed in Table 1 were employed. The PCR reaction mixture (100 µl total volume) consisted of 8 µl of 2.5 mM dNTPs , 10 µl of 10X PCR buffer, 8 µl of 25 mM $MgCl_2$, 1 µl of each 50 µM primer , 200 ng of cDNA and 2.5 u of Taq polymerase. PCR amplifications were carried out in a PTC-100-96V thermal cycler (MJ Research, Watertown, MA). The amplification program was: 95°C for 5 minutes followed by 30 cycles of 94°C for 1 minute, 55°C for 2 minutes, 72°C for 2 minutes and completed with a final extension at 72°C for 5 minutes. The resulting amplified DNA was electrophoresed on a 2% agarose gel and the correct sized bands (~400 bp) were isolated from the gel using Geneclean III (Bio 101, Vista, CA).

Library construction

In order to construct the heavy chain library the gel purified DNA containing the immunoglobulin heavy chain variable region genes was reamplified by PCR using primers containing the restriction sites Sfi I and Not I (Table 1). The reamplified products were digested with Sfi I and Not I (New England Biolabs, Beverly, MA) and gel purified as previously described. The purified heavy chain variable region fragments were ligated into the Sfi I and Not I digested phagemid vector pCANTAB-5E (Pharmacia Biotech, Uppsala, Sweden). Preparation of *E. coli* TG1 (Pharmacia Biotech, Uppsala, Sweden) competent cells was carried out as follows. Single colony of TG1 on minimal medium agar plate was inoculated in to 50 mL of LBG medium (LB medium, 20 mM glucose) and grown to an A_{600} of 0.5. Cells were pelleted and resuspended in 5 mL of ice-cold TSS (50 mg of bacto-tryptone, 25 mg of bacto-yeast extract, 25 mg of NaCl, 500 mg of PEG [M.W.3350], 250 µl DMSO and 250 µl of 1 M $MgCl_2$, pH 6.5). Ligated phagemid vector pCANTAB-5E plus the heavy chain variable regions were transformed into TG1 competent cells then selected on SOBAG (SOB medium, 100 µg/mL ampicillin and 2% glucose) agar plates.

Expression phage library

To prepare a library of expression phage, ampicillin selected cells were scraped from SOBAG agar plates and diluted to an A_{600} of 0.3 in 10 mL of 2X YT-AG medium (2X YT medium, 100μg/mL ampicillin, 2% glucose). The cultures were incubated at 37°C for 1 hour prior to adding 4 x 10^{10} pfu of M13KO7 phage (Pharmacia Biotech, Uppsala, Sweden) and additional incubation at 37°C for 1 hour. The cells were pelleted and resuspended in 10 mL of 2X YT-AK medium (2X YT medium, 100 μg/mL ampicillin, 50 μg/mL kanamycin). The cultures were incubated at 37°C overnight prior to collecting the supernatant containing the recombinant phage expressing the heavy chain variable region genes. Phage particles were precipitated with PEG/NaCl (0.2 g/mL polyethylene glycol 8000, 2.5 M NaCl) and resuspended in 10 mL of 2X YT medium with 3% BSA.

Panning selection of phage library

HPA-1a positive platelets were solubilized into 0.1 M $NaHCO_3$, 1 % Triton X-100, pH 9.6 (1 x 10^7/ mL) and 100 μl of this solution was added into microplate wells 16 hour at 4 C. After washing with phosphate-buffered saline (PBS)-0.05%Tween 20, each well was blocked with 300 μl of PBS-3% BSA and incubated at 37°C for 2 hours. PEG precipitated and resuspended phage particle were incubated for 30 minutes in PBS-3% BSA to reduce non-specific binding, then 100 μl of phage suspension was added into microtiter well coated with HPA-1a homozygous platelet lysate. The microplates were incubated for 2 hours at 37°C and then washed 20 times with PBS and 20 times with PBS-0.05% Tween 20. To rescue the captured phage, 100 μL of log-phase TG1 cells in 2X YT medium were added into each well and incubated at 37°C for 1 hour. Ampicillin (final 100 μg/mL), glucose (final 2%) and 4 x 10^{10} pfu of M13KO7 phage were added to TG1 cells recovered from each well. The cultures were incubated at 37°C for 1 hour, pelleted and re-suspended in 10 mL of 2X YT-AK medium. After incubation at 37°C overnight, the supernatant containing the selected recombinant phage was collected. Phage particle were precipitated with PEG/NaCl and resuspended in 10 mL of 2X YT-3% BSA medium. This panning selection step was repeated three times. After the third panning selection, selected phage were reinfected into TG1 and plated onto SOBAG plate. Single colonies were cultured separately in microplates and heavy chain variable region fragments were expressed on the surface of phage as previously described.

Preparation of microtiter plates for ELISA

Platelets from HPA-1a and HPA-1b homozygous donors were isolated from the platelet rich plasma by centrifugation at 1000 g for 15 minutes. Platelets were washed in PBS (pH 7.4) and residual red cells were removed by red cell lysis buffer. Platelet count was adjusted to 10^7 platelets / mL and then solubilized by incubation for 30 minutes at 4°C with PBS - 1% Triton X-100. Microplates were coated with 100 µl of platelet lysate diluted 1:1 in 0.05 M Sodium Carbonate buffer (pH 9.6) overnight at 4°C. Plates were washed with PBS-0.05 % Tween-20 and blocked with 300 µl of PBS-3% BSA at 37°C for 1 hour.

ELISA for platelet antigens against GP Ib/IX, IV and HLA were obtained from commercially available solid phase kit (GTI, Brookfield, WI). Recombinant glutathione S transferase (GST) fusion protein containing the N-terminus of GP IIIa (Barron Casella EA et al., *Blood*, 84:1157-63 (1994)), purified human GP IIIa (obtained from our research labs), and GP IIb/IIIa (Calbiochem, San Diego, CA) were applied to microtiter wells at a final concentration of 0.25 µg/well in 0.05 M sodium carbonate buffer. Reduced GP IIIa was obtained by incubating treated wells with 0.01 % β-mercaptoethanol for 5 minutes prior to blocking. Expression phage were standardized to a concentration of 10^9 phage/mL and 100 µL was added to each well for 2 hour at 37°C. After rinsing six times with PBS-0.05% Tween 20, bound phage were detected with 100 µL of biotinylated anti-M13 phage antibody at 20 µg/mL (5 prime-3 prime, Boulder, CO) in PBS, an alkaline phosphatase conjugated streptavidin (Vector Labs, Burlingame, CA), and absorbance read at 405 nm. Following the addition of p-nitrophenylphosphate(PNPP), in a Bio Tek EL340 microplate reader (Winooski, VT). We used human serum known to react with HPA-1a (biotinylated anti-human IgG was used for detection) as a positive control to verify antigen on the microplate. Our negative control consisted of an expression phage known to contain an human insert (verified by PCR) but expressing a protein not recognized by platelet antigens. Clones with the strongest absorbance were selected for further investigation and sequencing.

Flow cytometry characterization of display phage clones

Platelet rich plasma was isolated from 5 mL of anticoagulated whole blood from HPA-1a and HPA-1b homozygous donors. Platelets were washed in Tyrodes buffer and red cells removed with red cell lysis buffer. 10^6 platelets were incubated with 5 x 10^6

expression phage particles for 30 minutes at 37°C and then washed in Tyrodes buffer. Bound phage were detected with a biotinylated anti-M13 phage antibody (5 prime-3 prime, Boulder, CO) at a final concentration of 20 µg/mL (30 minutes at 37°C) and a streptavidin/fluorescein conjugate (4 µg/mL final concentration, 30 minutes at 4°C in the dark). For two-color analysis, a phycoerythrin (PE)-labeled antibody to CD 41 (Immunotech, Westbrook, ME) was added (4°C for 30 minutes in the dark). FITC (FL1) and PE (FL2) labeled platelets were analyzed by a Becton Dickinson FACScan flow cytometer (San Jose, CA) equipped with a 15 mW, 488 nm argon laser. Logarithmic scales were used to measure the mean fluorescence intensity of 10,000 events on cells expressing CD 41. Data analysis was performed on a Macintosh computer with Becton Dickinson software. A FITC-labeled antibody to CD 61 (Becton Dickinson, San Jose, CA) was used as a positive control. Our negative control was an expression phage known to contain a human insert but expressing a protein not specific for platelet antigens.

Nucleotide sequence analysis

Cultured phagemid vector DNA was isolated from infected TG1 cells containing human heavy chain fragment gene using phenol chloroform extraction. The DNA sequence was determined on an Applied Biosystems 373/377 automated DNA Sequencer (Applied Biosystems, Foster City, CA) and confirmed using a ^{35}S sequencing kit (Amersham, Cleveland, OH). The nucleotide and deduced amino acid sequence were analyzed using DNAsis software (Hitachi, San Bruno, CA). Identified sequences were compared against known sequences in the NCBI database.

Antibodies to the human heavy chain antibody polypeptide

According to the invention, the polypeptides may be produced recombinantly or by chemical synthesis, and fragments or other derivatives or analogs thereof, including fusion proteins, may be used as an immunogen to generate antibodies that recognize the heavy chain antibody polypeptide. Such antibodies include but are not limited to polyclonal, monoclonal, chimeric, single chain, Fab fragments, and a Fab expression library.

Pharmaceutical Compositions

The polypeptides and nucleic acid sequences of the present invention, such as SEQ. ID NO: 1 and SEQ. ID NO: 2, may be made into pharmaceutical compositions

with appropriate pharmaceutically acceptable carriers or diluents, such as a macromolecule, which is soluble in the circulatory system and which is physiologically acceptable where physiological acceptance means that those skilled in the art would accept injection of said carrier into a patient as part of a therapeutic regime. The carrier preferably is relatively stable in the circulatory system with an acceptable plasma half-life for clearance. Suitable carriers include, but are not limited to, water, alcoholic/aqueous solutions, emulsions, or suspensions, including saline and buffered media, and proteins such as serum albumin, heparin, immunoglobulin, polymers such as polyethylene glycol or polyoxyethylated polyols or proteins modified to reduce antigenicity by, for example, derivitizing with polyethylene glycol. Suitable carriers are well known in the art and are described, for example, in U.S. Patents 4,745,180, 4,766,106, and 4,847,325, and references cited therein. If appropriate, pharmaceutical compositions may be formulated into preparations including, but not limited to, solid, semi-solid, liquid, or gaseous forms, such as tablets, capsules, powders, granules, ointments, solutions, suppositories, injections, inhalants, and aerosols, in the usual ways for their respective route of administration. Methods known in the art can be utilized to prevent release or absorption of the composition until it reaches the target organ or to ensure time-release of the composition. A pharmaceutically-acceptable form should be employed which does not ineffectuate the compositions of the present invention. In pharmaceutical dosage forms, the compositions may be used alone or in appropriate association, as well as in combination with, other pharmaceutically-active compounds.

Accordingly, the pharmaceutical compositions of the present invention can be delivered via various routes and to various sites in an animal body to achieve a particular effect. Local or system delivery can be accomplished by administration comprising application or instillation of the formulation into body cavities, inhalation, or insufflation of an aerosol, or by parenteral introduction, comprising intramuscular, intravenous, peritoneal, subcutaneous intradermal, as well as topical administration.

Additionally, polypeptides of the invention may be used to avoid potential undesired blood coagulation that may arise from use of medical implementation, e.g. an indwelling device such as a catheter, stent, etc. In one preferred method, the implementation can be treated with a polypeptide of the invention (e.g. as a 1mg/ml

saline solution) prior to contact with a body fluid. Alternatively, or in addition, a polypeptide of the invention can be combined with the body fluid in an amount sufficient to minimize undesired blood coagulation.

The pharmaceutical compositions of the present invention can be provided in unit dosage form, wherein each dosage unit, e.g., a teaspoon, tablet, solution, or suppository, contains a predetermined amount of the composition, alone or in appropriate combination with other pharmaceutically-active agents. The term "unit dosage form" refers to physically discrete units suitable as unitary dosages for human and animal subjects, each unit containing a predetermined quantity of the composition of the present invention, alone or in combination with other active agents, calculated in an amount sufficient to produce the desired effect, in association with a pharmaceutically-acceptable diluent, carrier (e.g., liquid carrier such as a saline solution, a buffer solution, or other physiological aqueous solution), or vehicle, where appropriate. The specifications for the novel unit dosage forms of the present invention depend on the particular effect to be achieved and the particular pharmacodynamics associated with the pharmaceutical composition in the particular host.

Additionally, the present invention specifically provides a method of administering soluble constructs of the invention to a host, which comprises administering the composition of the present invention using any of the aforementioned routes of administration or alternative routes known to those skilled in the art and appropriate for the particular application. The "effective amount" of the composition is such as to produce the desired effect in a host which can be monitored using several end-points known to those skilled in the art. For example, one desired effect might comprise effective nucleic acid transfer to a host cell. Such transfer could be monitored in terms of a therapeutic effect, e.g., alleviation of some symptom associated with the disease being treated, or further evidence of the transferred gene or expression of the gene within the host, e.g., using PCR, Northern or Southern hybridization techniques, or transcription assays to detect the nucleic acid in host cells, or using immunoblot analysis, antibody-mediated detection, or particularized assays, as described in the examples, to detect protein or polypeptide encoded by the transferred nucleic acid, or impacted level or function due to such transfer. These

methods described are by no means all-inclusive, and further methods to suit the specific application will be apparent to the ordinary skilled artisan.

Furthermore, the amounts of each active agent included in the compositions employed in the examples described herein, i.e., add range, provide general guidance of the range of each component to be utilized by the practitioner upon optimizing the method of the present invention for practice either in vitro or in vivo. Moreover, such ranges by no means preclude use of a higher or lower amount of a component, as might be warranted in a particular application. For example, the actual dose and schedule may vary depending on whether the compositions are administered in combination with other pharmaceutical compositions, or depending on individual differences in pharmacokinetics, drug disposition, and metabolism. Similarly, amounts may vary in vitro applications depending on the particular cell line utilized, e.g., the ability of the plasmid employed for nucleic acid transfer to replicate in that cell line. Furthermore, the amount of nucleic acid to be added per cell or treatment will likely vary with the length and stability of the nucleic acid, as well as the nature of the sequence, and is particularly a parameter which needs to be determined empirically, and may be altered due to factors not inherent to the method of the present invention, e.g., the cost associated with synthesis, for instance. One skilled in the art can easily make any necessary adjustments in accordance with the necessities of the particular situation.

The therapeutic methods of the invention, as discussed above, generally include administration of an effective amount of a polypeptide or nucleic acid to a subject in need thereof. Typical subjects are mammals, particularly primates such as humans. Suitable subjects for treatment with a polypeptide or nucleic acid of the invention include persons or other mammals suffering from or susceptible to (i.e. prophylactic treatment) a disorder discussed above, e.g. a subject that is suffering from or susceptible to arterial or venous cardiovascular disorders; cerbrovascular thromboembolic disorders such as myocardial infarction; stroke; unstable angina; ischmeic sudden death; transient ischemic attack; atherosclerosis; reocculsion and restonosis after cardiac angioplasty or other surgery; thrombus and/or platelet-related problems; and the like.

As discussed above, polypeptides of the invention are suitably employed for various diagnostic applications, particularly to identify HPA-1b homozygous individuals that have a higher risk for developing NAIT and PTP, subjects that have the GPIIIa leu 33 risk factor, and for isolating autoantigens involved in HIV induced immune thromboctopenia that can be used for diagnostic purposes. For diagnostic applications, polypeptides of the invention can be either detectably labeled or unlabelled. For example, a variety of labels may be suitably employed to detectably label the polypeptide, such as radionuclides, fluors, enzymes, etc.

Anti-idiotypic antibodies can be prepared from polypeptides of the invention in accordance with known procedures. See, for instance, the procedures disclosed in E. Harlow et al., *Antibodies A Laboratory Manual* (Cold Spring Harbor Laboratory 1988). As discussed above, such anti-idiotypic antibodies are useful for therapeutic applications, including to replace human immunoglobulin therapy for treatment of immune thrombocytopenia.

The following examples further illustrate the present invention, and of course, should not be construed as in any way limiting its scope.

EXAMPLE 1

This example depicts the construction of a human Immunoglobulin heavy chain library. Immunoglobulin genes from cDNA from human PBMCs were amplified by PCR. Oligonucleotide primers for amplification of immunoglobulin heavy chain genes were chosen based on conserved DNA sequence found in the framework 1 (FR1) segment (5' primer) and the framework 4 (FR4) segment (3' primer) of the variable regions gene (Orlandi R. et al., *Proc. Natl. Acad. Sci USA*, 86:3833-7 (1989) (Table 1).

Table 1

Primers Used For PCR Amplification Of Human Immunoglobulin Heavy Chain Gene

Name of Primer	Sequence of Primers
VH 1-5'	5'-SAG GTG CAG CTG KTG SAG TCT GG-3' (SEQ ID NO:3)
VH 2-5'	5'-CAG GTR CAG CTG CAG SAG TCR GG-3' (SEQ ID NO:4)
VH 3-3'	5'-TGA RGA GAC GGT SAC CRK KGT BCC-3' (SEQ ID NO:5)
VH 4-3'	5'-ACC TAR RAC GGR SAS CTK GGT CCC-3' (SEQ ID NO:6)
Primer Containing Restriction Site	
VH 1-Sfi I	5'-GTC CTC GCA ACT GCG GGC CCA GCC GGC CAT GGC CSA GGT GCA GCT GKT GSA GTC TGG-3' (SEQ ID NO:7)
VH 2-Sfi I	5'-GTC CTC GCA ACT GCG GGC CCA GCC GGC CAT GGC CCA GGT RCA GCT GCA GSA GTC RGG-3' (SEQ ID NO:8)
VH 3-Not I	5'-GAG TCA TTC TCG ACT TGC GGC CGC TGA RGA GAC GGT SAC CRK KGT BCC-3' (SEQ ID NO:9)
VH 4-Not I	5'-GAG TCA TTC TCG ACT TGC GGC CGC TGA RGA GAC GGT GAC CRT KGT CCC-3' (SEQ ID NO:10)

R:A/G S:G/C K:G/T B:G/T/C

Mixed nucleotide bases were used to cover most of the frequent framework sequences. In order to construct the heavy chain variable region libraries containing immunoglobulin genes with specificity to HPA-1a protein, HPA-1a alloimmunized individuals were chosen as mononuclear cell donors. First-strand cDNA was synthesized from mRNA isolated from patient PBMC and used as a template for PCR amplification of immunoglobulin heavy chain variable region genes. Bands of the expected DNA size (~380 bp) were observed following agarose gel electrophoresis of PCR products. After the second round of amplification, PCR products were purified by gel electrophoresis and digested by restriction enzyme to get cohesive ends. Heavy chain variable region genes were ligated into phagemid vector pCANTAB-5E and transformed into *E. coli* TG1.

EXAMPLE 2

This example depicts library screening by panning.

Phage particles that expressed immunoglobulin heavy chain variable region fragment were screened by a panning method on microplates coated with HPA-1a platelet membrane protein. After three rounds of panning selection, expression phage were analyzed by ELISA on microplates coated with platelet lysate from HPA-1a homozygous and HPA-1b homozygous donors. Several phage that bound to HPA-1a protein were isolated, but these phage also bound to HPA-1b lysate. After three rounds of panning selection, only one clone appeared to have specificity for HPA-1a lysates relative to HPA-1b lysates.

EXAMPLE 3

This example depicts the binding specificity of an isolated anti-HPA-1a clone.

The clone (ML1) which appeared to have anti-HPA-1a specificity was analyzed further using ELISA microplate wells coated with different antigens (Table 2 below). Clone ML3, which was used as a negative control clone, contained a human immunoglobulin insert but did not bind to the HPA-1a antigen.

Table 2

ELISA Assay Of Anti-HPA-1a Clone

	Mean OD_{405}		
Antigenic Target	Clone ML1	Clone ML3	Background
Platelet lysate from a HPA-1a homozygous donor #1	0.653	0.220	0.124
Platelet lysate from a HPA-1a homozygous donor #2	0.680	0.170	0.113
Platelet lysate from a HPA-1b homozygous donor	0.200	0.196	0.067
Platelet GPIIb/IIIa, HPA-1a form	0.718	0.175	0.103
Platelet GPIIIa, HPA-1a form	0.441	0.200	0.141
Reduced platelet GPIIIa, HPA-1a form	0.136	0.152	0.123
Recombinant GST N-terminal GPIIIa, HPA-1a form	0.616	0.158	0.099
Platelet GPIb/IX	0.067	0.100	0.072
Platelet GPTV	0.113	0.125	0.094
HLA antigen	0.093	0.125	0.076

Clone ML1 bound to the platelet lysates from two HPA-1a homozygous donors but did not bind to lysates from a HPA-1b homozygous donor. These results show that clone ML1 binds specifically to platelet lysate or glycoproteins carrying the HPA-1a epitope. The difference in signal between HPA-1a and HPA-1b was not due to different levels of antigen as demonstrated by comparable signal using the anti-GPIIIa antibody

control (data not shown). Furthermore, this clone bound to the HPA-1a form of purified platelet GPIIIa and a GST-GPIIIa N-terminus fusion protein. It did not bind to β-mercaptoethanol reduced purified platelet GP IIIa (HPA-1a form) suggesting that the immunoglobulin heavy chain antibody fragment produced by this recombinant phage recognized a conformational HPA-1a epitope formed by disulfide bonds. Clone ML1 did not bind to other platelet glycoproteins such as GPIb/IX, GPIV, or HLA antigens.

EXAMPLE 4

This example depicts flow cytometry results.

Figure 1 of the drawings shows the flow cytometry results when expression phage particles were tested against HPA-1a or HPA-1b homozygous platelets. Clone ML3 was used as a negative control clone. Results are shown as histograms comparing the reactivity to HPA-1a positive (histogram B) and HPA-1a negative (histogram A) platelets to clone ML3 incubated with HPA-1a homozygous platelets (negative control histogram). Specific binding of clone ML1 to HPA-1a platelets relative to the HPA-1b platelets was observed. These results confirm the specificity of clone ML1 for the HPA-1a epitope of GP IIIa.

EXAMPLE 5

This example depicts the sequence analysis of HPA-1a binding heavy chain antibody fragment .

Nucleotide sequence of the selected clone was determined by an automated DNA sequencer and confirmed with a ^{35}S sequencing kit (Figure 2 (SEQ. ID NO: 1)). Sequence analysis revealed that the inserted heavy chain variable region gene was 378 base pairs in length. Amino acid sequence (SEQ. ID NO: 2) was deduced from the nucleic acid sequence and then compared with other data base and reported sequences (Table 3 below).

Table 3

Comparison of Deduced Protein Sequence of VH Genes of Antibody Fragment

GenBank Access. No.	FR 1	COR 1	FR 2	COR 2	FR 3	COR 3	FR 4
ML1	QVQLQESGPGLVKPSETLSLTCNVSG GSIS	SYYWS	WIRQPPGKGLEWLG	YLYNSGSTIYSPALES	RATISVDTSKNQFSLKLSSYTAADTA VYYCAR	PEPYSSGHYRGIFD	AFDIWGQGTKVTVLG
S80752[a]	---VQ--AEVKR-GAAVKVS-KA--YRFT	GH-MH	-V--A--Q---M-	WINPNSGGTSYAQKFQG	-V-MTR---ISTAYMEMTRLRYD------*	AGGLGGYY-YAMNI	****----T---SS
S55017[b]	-L----------T------	SS---G	---------I-	SI-Y---Y-N-S-K-	-V----------	*RKD----S-YP-	---------M---SSG
S50735[c]	----------T------	-----	----------I-	-I-Y---N-N-S-K-	-V----------	DRSLTRHY-DSSG	---------M---SSG
U00486[d]	---R----------T-----	-----	----------I-	-I-Y---N-N-S-K-	-V----------G	GRLFIVATIG	*--Y----L---SS
U00493[e]	----------T------	-----	----------I-	-I-Y---N-N-S-K-	-V----------	VRGVRGVIIRQR	---------M---SS
S67826[f]	----------T---D---	-----	-----RA---I-	-I-HG---N---S-K-	-V----------R---------M----	DRHC-G-TCY-	*M-V----T---SSG

Identical residues in selected genes are represented by hyphens. Deficient residues in selected genes are represented by star.

FR, framework region; CDR, complementary determining region.

[a] anti-HPA 1a antibody heavy chain variable region gene.

[b] IgM VH4 immunoglobulin variable heavy chain gene.

[c] anti-lipid A antibody immunoglobulin M heavy chain V region.

[d] Human immunoglobulin heavy chain variable region (clone Amu 1a3-3).

[e] Human immunoglobulin heavy chain variable region (clone Amu 1d3-3).

[f] Ig VH4 immunoglobulin heavy chain variable region.

This comparison indicates that this heavy chain variable region gene is contained in the VH4 family and showed high homology with IgM variable region gene. Comparison with a previously reported anti-HPA-1a heavy chain variable region gene (Ouwehand et al., *Blood* 1995; 86:4430-6) did not reveal detectable homology suggesting that this clone encoded a different heavy chain antibody fragment.

EXAMPLE 6

This example demonstrates the ability of the isolated expression bacteriophage with human DNA insert to alter platelet aggregation. (see Figure 3).

HPA-1a platelets were incubated with clone ML1 for 1 hour at room temperature. Aggregation was evaluated using standard procedures following addition of collagen (10ug/mL) on a Chronolog four channel aggregometer. Figure 3, Curve A, shows inhibition of collagen-induced aggregation following incubation with phage ML1. Figure 3, Curve B, shows normal aggregation following incubation with a negative control clone and Curve C shows normal aggregation.

All of the references cited herein, including the published literature, patents, and PCT applications, are hereby incorporated in their entireties by reference.

While this invention has been described with emphasis upon preferred embodiments, it will be obvious to those of ordinary skill in the art that the preferred embodiments can be modified and varied. It is intended that the invention can be practiced otherwise than specifically described herein. Accordingly, this invention includes all modifications encompassed within the spirit an scope of the appended claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION

(i) APPLICANT:

(ii) TITLE OF THE INVENTION: IDENTIFICATION OF HUMAN HEAVY CHAIN ANTIBODY FRAGMENT DIRECTED AGAINST HUMAN PLATELET ALLOANTIGEN 1A (HPA-1A)

(iii) NUMBER OF SEQUENCES: 10

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Dike, Bronstein, Roberts & Cushman, LLP
(B) STREET: 130 Water Street
(C) CITY: Boston
(D) STATE: MA
(E) COUNTRY: USA
(F) ZIP: 02109

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Diskette
(B) COMPUTER: IBM Compatible
(C) OPERATING SYSTEM: DOS
(D) SOFTWARE: FastSEQ for Windows Version 2.0

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: 60/048,645
(B) FILING DATE: 05-JUN-1997

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Corless, Peter F
(B) REGISTRATION NUMBER: 33,860
(C) REFERENCE/DOCKET NUMBER: 48396-PCT

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 617-523-3400
(B) TELEFAX: 617-523-6440
(C) TELEX:

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 126 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

```
Gln Val Gln Leu Gln Glu Ser Gly Pro Gly Leu Val Lys Pro Ser Glu
 1               5                  10                  15
Thr Leu Ser Leu Thr Cys Asn Val Ser Gly Gly Ser Ile Ser Ser Tyr
            20                  25                  30
Tyr Trp Ser Trp Ile Arg Gln Pro Pro Gly Lys Gly Leu Glu Trp Leu
        35                  40                  45
Gly Tyr Leu Tyr Asn Ser Gly Ser Thr Ile Tyr Ser Pro Ala Leu Glu
    50                  55                  60
Ser Arg Ala Thr Ile Ser Val Asp Thr Ser Lys Asn Gln Phe Ser Leu
65                  70                  75                  80
Lys Leu Ser Ser Val Thr Ala Ala Asp Thr Ala Val Tyr Tyr Cys Ala
                85                  90                  95
Arg Pro Glu Pro Tyr Ser Ser Gly Trp Tyr Arg Gly Ile Phe Asp Ala
            100                 105                 110
Phe Asp Ile Trp Gly Gln Gly Thr Lys Val Thr Val Leu Gly
        115                 120                 125
```

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 384 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

```
ATGGCCCAGG TGCAGCTGCA GGAGTCGGGC CCAGGACTGG TGAAGCCTTC GGAGACCCTG     60
TCCCTCACCT GCAATGTCTC TGGTGGCTCC ATCAGTAGTT ACTACTGGAG TTGGATCCGG    120
CAGCCCCCAG GAAGGGACTG GAGTGGCTTG GGTATTTGTA TAACAGTGGC AGCACCATCT    180
ACAGCCCCGC CCTCGAGAGT CGAGCCACCA TATCCGTAGA CACGTCCAAG AACCAGTTCT    240
CCCCTGAAGC TGAGCTCTGT GACCGCCGCA GACACAGCTG TGTATTACTG TGCGAGGCCC    300
GAACCTTATA GCAGTGGCTG GTACCGGGGC ATTTTTGATG CTTTTGATAT CTGGGGCCAA    360
GGGACCAAGG TCACCGTCCT AGGT                                           384
```

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

```
SAGGTGCAGC TGKTGSAGTC TGG                                           23
```

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

```
CAGGTRCAGC TGCAGSAGTC TGG                                           23
```

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

TGARGAGACG GTSACCRKKG TBCC 24

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

ACCTARRACG GRSASCTKGG TCCC 24

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 57 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

GTCCTCGCAA CTGCGGGCCC AGCCGGCCAT GGCCSAGGTG CAGCTGKTGS AGTCTGG 57

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 57 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

GTCCTCGCAA CTGCGGGCCC AGCCGGCCAT GGCCCAGGTR CAGCTGCAGS AGTCRGG 57

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 48 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

GAGTCATTCT CGACTTGCGG CCGCTGARGA GACGGTSACC RKKGTBCC 48

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 48 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

GAGTCATTCT CGACTTGCGG CCGCTGARGA GACGGTGACC RTKGTCCC 48

WHAT IS CLAIMED IS:

1. A molecule comprising a nucleic acid sequence of SEQ. ID NO: 1.

2. A molecule comprising a nucleic acid that encodes SEQ ID NO:2.

3. A molecule comprising an amino acid sequence of SEQ. ID NO: 2, or allelic variants or analogs, including fragments thereof.

4. A pharmaceutical composition comprising a nucleic acid sequence of claim 1 or 2 and a pharmaceutically acceptable carrier.

5. A pharmaceutical composition comprising a polypeptide of claim 2 and a pharmaceutically acceptable carrier.

6. A polypeptide that binds specifically to HPA-1a.

7. A polypeptide that binds with at least about a 2-fold greater specificity for HLA-1a than for HLA-1b.

8. A polypeptide that binds with at least about a 3-fold greater specificity for HLA-1a than for HLA-1b.

9. A polypeptide having the binding affinity for HPA-1a about equal to or greater than ML1.

10. An antibody having the identifying characteristics of ML1.

11. An antibody that is ML1.

12. A molecule, polypeptide or antibody according to any one of claims 3 or 6-11, which is a monoclonal antibody.

13. A molecule, polypeptide or antibody according to any one of claims 3 or 6-11, which is a polyclonal antibody.

14. A polypeptide comprising an amino acid sequence that has at least about 70 percent sequence identity to SEQ ID NO:2.

15. A polypeptide comprising an amino acid sequence that has at least about 80, 85, 90, 95, 97 or 98 percent sequence identity to SEQ ID NO:2.

16. A molecule, polypeptide or antibody of any one of claims 1-15 that is of human origin.

17. A molecule, polypeptide or antibody of any one of claims 1-16 that is completely of human origin.

18. A molecule comprising a nucleic acid sequence that has at least about 70 percent sequence identity to SEQ. ID NO: 1.

19. A molecule comprising a nucleic acid sequence that has at least about 80, 85, 90, 95, 97 or 98 percent sequence identity to SEQ. ID NO: 1.

20. A molecule comprising a nucleic acid sequence comprising at least 20 base pairs and that hybridizes to SEQ ID NO:1 under normal stringency conditions.

21. A molecule comprising a nucleic acid sequence comprising at least 20 base pairs and that hybridizes to SEQ ID NO:1 under high stringency conditions.

22. A method of treating or preventing thrombus or a platelet-related disorder comprising administering to a subject an effective of a molecule, polypeptide or antibody of any of claims 1-21.

23. A molecule of claim 1 comprising a reporter group selected from the group consisting of a radiolabel, a fluorescent label, an enzyme, a substrate, a solid matrix, and a carrier.

24. A molecule of claim 3 comprising a reporter group selected from the group consisting of a radiolabel, a fluorescent label, an enzyme, a substrate, a solid matrix, and a carrier.

25. A method of protecting against or treating diseases involving platelet aggregation, wherein said method comprises administering a molecule, polypeptide or antibody any one of claims 1-21 pharmaceutically acceptable carrier.

26. The method of claim 25 wherein an effective amount of a molecule, polypeptide or antibody of any one of claims 1-21 is administering to a subject suffering from or susceptible to a disease involving platelet aggregation.

27. A method of inhibiting binding of fibrinogen to blood platelets comprising administering to a subject an effective amount of a molecule, polypeptide or antibody any of one of claims 1-21.

28. A method for treatment or prevention of a disease that is an arterial or venous cardiovascular disorders, myocardial infarction, stroke, unstable angina, ischemic sudden death, transient ischemic attack, atherosclerosis, or undesired reocculsion and restonosis after surgery, comprising administering to a subject suffering from or susceptible from such disease an effective amount of a molecule, polypeptide or antibody of any one of claims 1-21.

29. A method for isolating autoantigens involved in HIV-induced immune thrombocytopenia, comprising contacting a body fluid or tissue sample with a molecule, polypeptide or antibody of any one of claims 3 or 6-15.

30. An anti-idiotypic antibody of a molecule, polypeptide or antibody of any one of claims 3 or 6-15.

31. A method of any one of claims 3 or 6-15 wherein the molecule, polypeptide, antibody or nucleic acid is co-administered with another pharmaceutical agent.

32. A method of claim 31 wherein the molecule, polypeptide, antibody or nucleic acid is linked to the pharmaceutical agent.

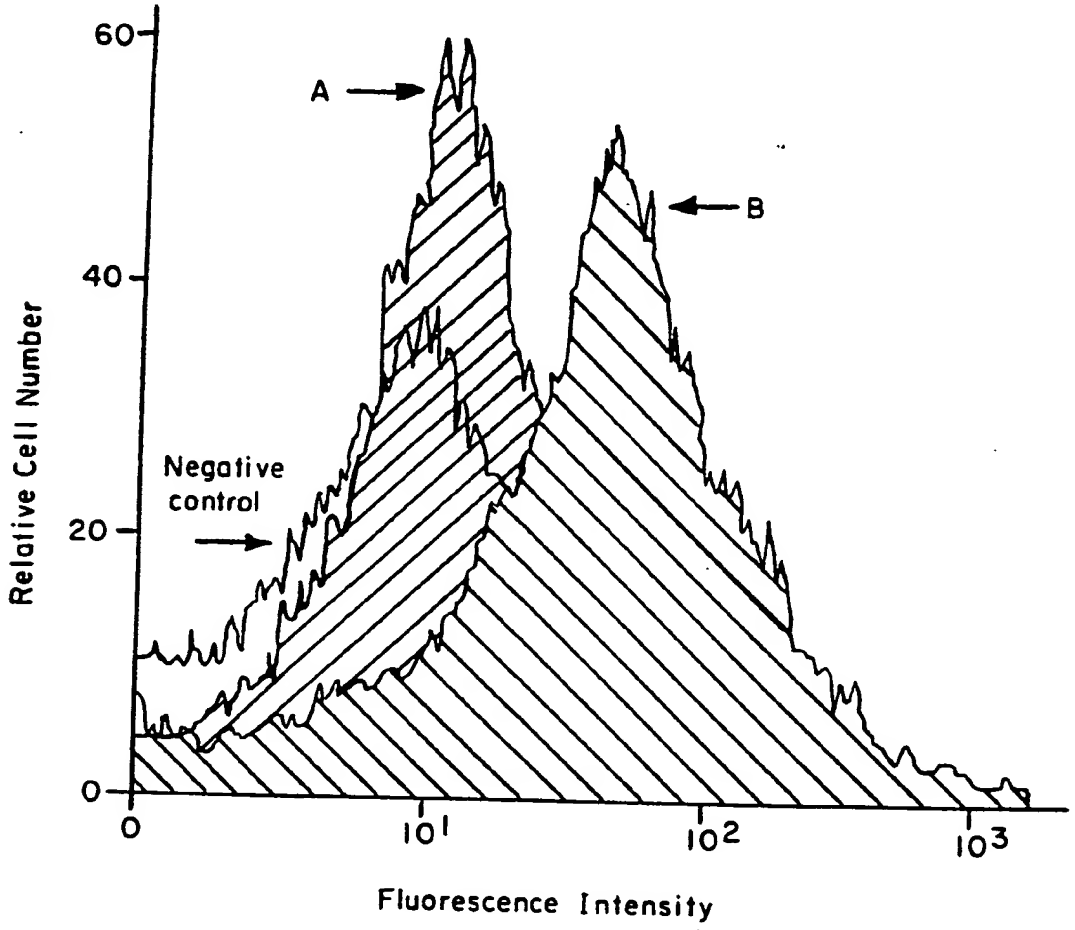
60
40
20
0
Relative Cell Number
A
B
Negative control
0
10¹
10²
10³
Fluorescence Intensity

FIG. 1

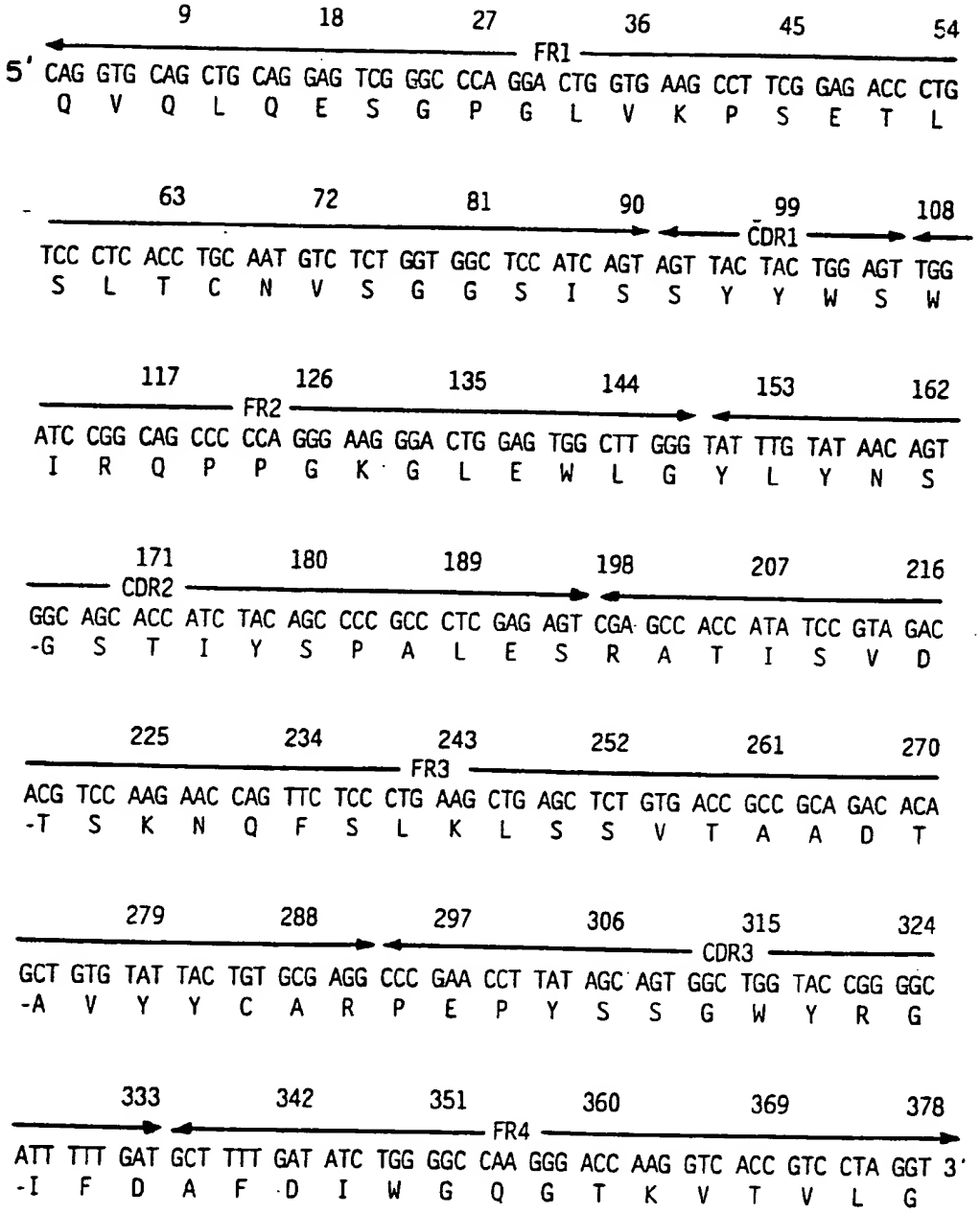
9 18 27 36 45 54
FR1
5' CAG GTG CAG CTG CAG GAG TCG GGC CCA GGA CTG GTG AAG CCT TCG GAG ACC CTG
Q V Q L Q E S G P G L V K P S E T L
63 72 81 90 99 108
CDR1
TCC CTC ACC TGC AAT GTC TCT GGT GGC TCC ATC AGT AGT TAC TAC TGG AGT TGG
S L T C N V S G G S I S S Y Y W S W
117 126 135 144 153 162
FR2
ATC CGG CAG CCC CCA GGG AAG GGA CTG GAG TGG CTT GGG TAT TTG TAT AAC AGT
I R Q P P G K G L E W L G Y L Y N S
171 180 189 198 207 216
CDR2
GGC AGC ACC ATC TAC AGC CCC GCC CTC GAG AGT CGA GCC ACC ATA TCC GTA GAC
-G S T I Y S P A L E S R A T I S V D
225 234 243 252 261 270
FR3
ACG TCC AAG AAC CAG TTC TCC CTG AAG CTG AGC TCT GTG ACC GCC GCA GAC ACA
-T S K N Q F S L K L S S V T A A D T
279 288 297 306 315 324
CDR3
GCT GTG TAT TAC TGT GCG AGG CCC GAA CCT TAT AGC AGT GGC TGG TAC CGG GGC
-A V Y Y C A R P E P Y S S G W Y R G
333 342 351 360 369 378
FR4
ATT TTT GAT GCT TTT GAT ATC TGG GGC CAA GGG ACC AAG GTC ACC GTC CTA GGT 3'
-I F D A F D I W G Q G T K V T V L G

FIG. 2

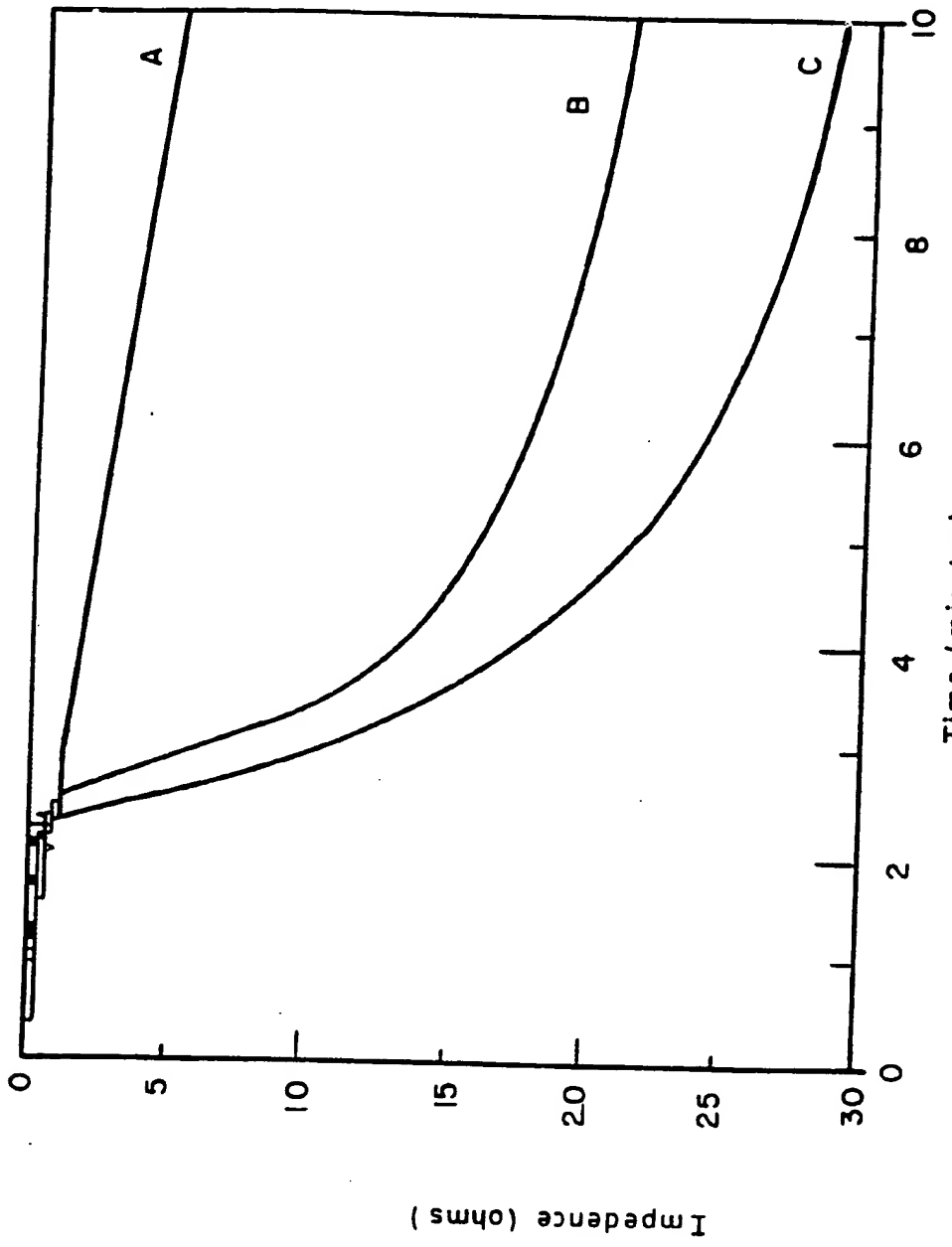

FIG. 3

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/11328

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A61K 39/395; C07K 16/34; C07H 21/04; C12N 15/13

US CL :Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/130.1, 131.1, 133.1, 141.1, 143.1, 153.1, 173.1, 178.1; 530/387.1, 387.2, 387.3, 388.1, 388.7, 389.6, 391.1; 536/23.53

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, DIALOG, BIOSIS, EMBASE, CA, MEDLINE, WPI, GENBANK

search terms: hpa-1a, hpa-1b, ml1, sequence id numbers 1 and 2, thrombus, platelet, cardiovascular, restenosis

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5,399,481 A (MCMILLAN et al.) 21 March 1995, see entire document.	1-15, 18-21, 23-24
Y	BARRON-CASELLA et al. Expression and Purification of Functional Recombinant Epitopes for the Platelet Antigens, $P1^{A1}$ and $P1^{A2}$. Blood. 15 August 1994, Volume 84, No. 4, pages 1157-1163, see entire document.	1-15, 18-21, 23-24
Y	WEISS et al. A Monoclonal Antibody (SZ21) Specific for Platelet GPIIIa Distinguishes $P1^{A1}$ from $P1^{A2}$. Tissue Antigens. 1995, Volume 46, pages 374-381, see entire document.	1-15, 18-21, 23-24

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

24 AUGUST 1998

Date of mailing of the international search report

02 OCT 1998

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer

PHILLIP GAMBEL

Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/11328

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 16, 17, 22, 25-28
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/11328

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

424/130.1, 131.1, 133.1, 141.1, 143.1, 153.1, 173.1, 178.1; 530/387.1, 387.2, 387.3, 388.1, 388.7, 389.6, 391.1; 536/23.53